



Antimicrobial and bacteriostatic activity of surfactants against *B. subtilis* for microbial cleaner formulation

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Abstract

Cleaning products containing live bacteria that form spores of *Bacillus* spp. as active substances are becoming increasingly common in probiotic cleaner formulation. The quality of cleaning performance for the production of probiotic cleaners does not only depend on the potential of the bacterial strains used, but also on the chemical components of the formulations. In this study, the surfactants and other additives were investigated as biocidal or bacteriostatic against *B. subtilis*, and the viability of *B. subtilis* was examined at different pH ranges for microbial cleaner formulation. As a result, it was discovered that the *B. subtilis*, which can be used in the microbial cleaner formulation, shows higher growth and viability at the neutral pH, and it passes into the death phase at pH 3. According to antagonistic activity results, the Gram-positive *S. aureus* and *K. pneumoniae* were the most sensitive bacteria while *B. cereus* was the most resistant bacteria. The anionic surfactants such as linear alkylbenzene sulfonic acid and sodium lauryl ether sulfate act as bacteriostatic on *Bacillus* spp. and do not cause cell death. In the view of these results, the usage of appropriate bacterial cultures and the correct stabilization of the formulations are also critical elements in the development of microbial cleaner formulations.

Keywords *B. subtilis* · Probiotics · Microbial based cleaning · Surfactants · Bacteriostatic activity · Antagonistic activity

Introduction

Nowadays, new-generation cleaning products containing probiotic bacteria or spores as active ingredients attract popular attention. These new-generation cleaning products are also called probiotic cleaners (Teasdale and Kademi 2018; Spöka et al. 2018). The purpose of these new generation probiotic cleaning products was to prevent the development of pathogenic species by tolerating the presence of probiotic species (Vandini et al. 2014). This approach suggesting to replace all pathogens with beneficial bacteria, may be more effective at reducing pollution (Caselli 2017). New-generation probiotic cleaning products contain beneficial probiotic bacteria that can compete with pathogenic species by consuming nutrients on contaminated surfaces. For this reason,

these probiotic species are preferred because they remain on the surface where they are applied and prevent colonization and pollution by pathogenic bacteria (Spöka et al. 2018). As a result of researches aiming at the production of probiotic cleaner, it was found that 100% of *Bacillus subtilis*, 79% of *Bacillus amyloliquefaciens*, 30% of *Bacillus amyloliquefaciens* and other *Bacillus* species were used in the cleaner formulation (Alkaya et al. 2017; Hong et al. 2008; Teasdale and Kademi 2018). The ability of *Bacillus* species to ferment in the acid, alkaline, and neutral pH ranges, combined with the wide temperature ranges, has led to the development of a variety of biotechnological applications with the desired temperature, pH activity, and stability properties (Schallmeyer et al. 2004).

To apply an antimicrobial effect, the agent must reach an effective concentration in the biochemical domain. The majority of antimicrobial agents does not affect the outer surface of bacteria. Therefore, the antimicrobial agent must be able to penetrate the bacteria to produce any damage. Gram-positive cells, which lack the additional lipopolysaccharide layer owned by gram-negative bacteria, are more sensitive to many antibacterial agents (Moore 1997; Reygaert 2018). Most of the antibacterial agents disrupt

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the bacterial cytoplasmic membrane and cell wall. Multiple mechanisms involving permeabilization of the bacterial membrane affect transport processes, intracellular substrate reservoirs, charge gradients, co-factor concentrations, ion gradients and nutrient concentration (Moore 1997). Antibacterial agents cause both bacteriostatic and bactericidal effects. Surfactants have been known to have significant antimicrobial activity for many years (Cowles 1938). Surfactants can be divided into cationic, anionic, amphoteric and nonionic groups. These compounds generally contain carboxylates, sulfonates, sulfates and phosphates as hydrophilic heads. Anionic surfactants are often used in different types of detergents, where the hydrophobic chain for optimum detergent consists of a linear chain of 12–16 carbon atoms (Wieczorek et al. 2018). The antimicrobial activity of surfactants are influenced not only by the chemical properties of surfactants, but also by how the target microorganism grows before and during surfactant treatment (Ishikawa et al. 2002).

Anionic surfactants have a negatively charged head group and the counter ion is usually Na^+ . This group includes traditional soaps (carboxylates) and the first synthetic detergents: sulfates and sulfonates. Sulphates and sulfonates are more advantageous than carboxylates because they can be used in hard water because they have more tolerance to divalent metal ions (Moore 1997). The bactericidal properties of anionic and cationic detergents were tested against Gram-positive and Gram-negative bacteria and their bactericidal properties were compared by Baker et al. (1941). In their study, it was concluded that anionics are effective only against Gram positive bacteria and are much less effective than cationics.

The alkyl sulfates are a combination of half-esters of sulfuric acid (Cowles 1938). Cowles (1938) examined the bacteriostatic properties of sodium alkyl sulfate with different carbon chain lengths against Gram-negative and Gram-positive bacteria. Linear alkylbenzene sulphonate (LAS) is an anionic surfactant (Ebede and Nwokoye 2019). LAS is a mixture that contains an aromatic ring sulphonated at the para position and attached to a linear alkyl chain (Ebede and Nwokoye 2019). LAS is an anionic surfactant used in detergents, such as laundry powders, dishwashing products and surface cleaner. LAS is a water-soluble, biodegradable and surface active surfactant (Ebede and Nwokoye 2019). The linear alkylbenzene sulfonates (LAS) constitute major group of synthetic surfactants that are used today (Brandt et al. 2001). Cowles (1938) described that the Gram-negative organisms are not prevented from growing by the concentrations of alkyl sulfates used, whereas the Gram-positives, for the most part, are inhibited.

Sodium lauryl sulfate (SLS) is an anionic surfactant. The antimicrobial properties of SLS, alone or in combination with other antimicrobials, against pathogens are well known (Byelashov et al. 2008). The antibacterial action of

SLS involves lysis of bacterial cells, denaturation of proteins and enzymes, damage of cell membranes and changes in cell permeability (Byelashov et al. 2008).

Cleaning products contain live bacteria that form spores like *Bacillus* spp., as active substances (Alkaya et al. 2017; Hong et al. 2008; Teasdale and Kademi 2018). The probiotic cleaning composition also includes one or more type of surfactants or other additives. Surfactants and other additives used in the cleaning composition containing probiotics should not have any negative effects on *Bacillus* species (Cowles 1938). These optional ingredients should be chosen so that they have little or no negative impact on the *Bacillus subtilis* (Brooke 2014). In this study, the surfactants and other additives were investigated as biocidal or bacteriostatic against *B. subtilis* for probiotic cleaner formulation.

Materials and methods

Materials

Nutrient broth (NB), Nutrient agar (NA), Luria–Bertani (LB), Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) were procured from Merck (Merck, Darmstadt, Germany). Polyethylene Glycol (PEG) 40, benzophenone, citric acid, isopropyl alcohol, mono propylene glycol, triethanol amine and nonionic, anionic and amphoteric surfactants were supplied from Hayat Kimya San. A. Ş. (Turkey). In all experiments, deionization water was used. Probiotic *Bacillus subtilis* ATCC 6633 and Gram positive bacteria *Bacillus cereus* ATCC 11778 and *Staphylococcus aureus* ATCC 6538 and Gram negative bacteria *Klebsiella pneumoniae* ATCC 43816 and *Salmonella typhimurium* ATCC 14028 were obtained from the culture collection of Algal Biotechnology and Bioprocess Laboratory of Yildiz Technical University (Turkey).

Production of pathogenic bacteria

Stock cultures of Gram positive *B. cereus* and *S. aureus* and Gram negative *K. pneumoniae* and *S. typhimurium* bacteria were maintained at $-20\text{ }^{\circ}\text{C}$ in nutrient broth (NB) medium, supplemented with 25% (v/v) glycerol. A loopful of the glycerol stock was transferred onto nutrient agar (NA) medium, streaked and incubated at $37\text{ }^{\circ}\text{C}$ for 24 h. Single colonies were inoculated overnight onto nutrient broth (NB) at $37\text{ }^{\circ}\text{C}$. After that, bacteria were spread on the nutrient agar (NA) plates for antagonistic activity assay (Vandini et al. 2014; Ndlovu et al. 2017).

Production of probiotic bacteria *B. subtilis*

The *B. subtilis* ATCC 6633 was maintained in 50% glycerol at $-80\text{ }^{\circ}\text{C}$. A loopful of the glycerol stock of probiotic

bacteria was activated by adding onto Luria Bertani (LB) liquid medium. Then, planting was done on Tryptic Soy Agar (TSA) solid media and Petri dishes were incubated at 37 °C for 24 h. Pure culture was obtained from the colonies grown in TSA Petri dishes. For this, single bacterial colonies were inoculated onto 10 mL LB medium and incubated at 37 °C for 24 h (Ndlovu et al. 2017; Vehapi and Özçimen 2020).

Determining the optimum growth temperature and media for *B. subtilis*

In this study, the effect of different temperatures (25 ± 2 °C, 30 ± 2 °C and 35 ± 2 °C) and growth media (LB and TSB) on the growth of *B. subtilis* was investigated. The single colony of *B. subtilis* was inoculated onto 10 mL LB and TSB liquid medium, and bacteria were incubated at different temperatures such as 25 ± 2 , 30 ± 2 and 35 ± 2 °C for 30 h, and the effect of temperature and growth media on bacterial growth was investigated. At regular intervals (e.g. 1 h), the measurements of optical density (OD) of a liquid sample at 600 nm was achieved using spectrophotometer and the optimum temperatures at which the bacteria grew was determined (Vandini et al. 2014; Vehapi and Özçimen 2020).

Growth rates of *B. subtilis* in buffered media

Bacillus subtilis was incubated at 25 and 30 °C for 30 h at various pH values from pH 3 to 8 in buffered Luria Bertani (LB) liquid nutrient medium and growth curves were obtained by optical density (OD) measurements at regular intervals (e.g. 1 h). The optical density (OD) of a liquid sample was measured at 600 nm using PG Instruments T-60 UV Visible Spectrophotometer device (Vehapi and Özçimen 2020).

Sporulation of *B. subtilis*

Spores of *Bacillus* spp. are more resistant to adverse environmental conditions such as high and low temperatures, chemicals and light, because bacteria do not show any metabolic activity. Viable spore counts from spore suspensions were evaluated by colony count after heat treated at 80 °C for 20 min (Nakata 1963; Chen et al. 2010).

Quantification of the microbial load

Counting was performed by preparing 10^{-5} , 10^{-6} and 10^{-7} dilutions of the test suspension in diluent buffer (PBS). A sample of 1 mL for each dilution was inoculated in duplicate using the spread plate technique. Each 0.1-mL sample was transferred into separate Petri dishes with 20 mL volume of melted TSA. The number of Colony-forming Units (CFU) was determined

by colony counting after incubation of TSA plates at 37 °C for 24 h. The numbers of *Bacillus* was expressed in CFU mL⁻¹ and the calculation of the microbial load was performed by using the following Eq. (1) (Prabakaran and Balaraman 2006; Vandini et al. 2014):

$$\text{CFU mL}^{-1} = (\text{CFU} \times \text{DF})/0.1 \text{ mL} \quad (1)$$

CFU colony-forming units, DF dilution factor.

The cultures that contain about 1×10^8 CFU mL⁻¹ were centrifuged at 12,000 rpm for 20 min to separate cell mass from the nutrient solution. After that, the cell mass was freeze-dried and stored before use for antagonistic activity assay (Prabakaran and Balaraman 2006).

Antagonistic activity of *B. subtilis*

Pathogenic bacteria were cultured in nutrient broth (NB) medium and incubated at 37 °C for 24 h. Nutrient agar (NA, 20 mL) was poured into each sterile petri dish (90 mm diameter). Suspensions (100 µL) of pathogenic bacteria cultured for 24 h were spread on the plates. The spores of *B. subtilis* was freeze-dried and stored before use for antagonistic activity assay. After that, *B. subtilis* was weighed as 0.01 mg and dissolved with 1 mL of phosphate-buffered saline (PBS), and its antagonistic effect against *B. cereus*, *S. aureus*, *K. pneumoniae* and *S. typhimurium* species was investigated by disc diffusion method. *B. subtilis* (30 µL) was directly impregnated into the paper discs (6 mm) of agar plates inoculated with indicator bacteria. The plates were incubated for 24 h at 37 °C, and the rates of the *Bacillus* spores germination and zone inhibition of pathogenic bacteria were measured in millimeters for 3 days (Lertcanawanichakul and Sawangnop 2008).

Antibacterial effect of surfactants against *B. subtilis*

The antibacterial effects of surfactants and other additives used for cleaner formulation were determined against *B. subtilis* probiotic bacteria by agar well diffusion method. 100 µL of bacterial culture of *B. subtilis* was spread onto pre poured TSA plates (20 mL/plate). After that, 30-µL surfactant solutions were impregnated on agar wells (approximate, 6 mm). Petri plates were incubated at 37 °C for 20 h, the clear zones around well were visually examined, and the thickness of the clear zone around well was measured with a millimeter scale. The analyses were made in three copies and by calculating the average values (Eswaranandam et al. 2004; Vehapi et al. 2020).

Growth rates in liquid batch cultures

The antimicrobial activities of anionic surfactants linear alkylbenzene sulfonic acid (LABSA) and sodium lauryl ether sulfate (SLES) against *B. subtilis* were determined with

respect to treatment time in culture medium (TSB) (Fig. 1). Surfactants at 5 and 10% concentrations were added at 2 h into TSB containing *B. subtilis*. For this, bacterial cell cultures were inoculated into TSB. After 2 h of inoculation, the surfactants were added onto TSB and at 1-h intervals for 24 h. OD was measured by spectrophotometer and incubated at 37 °C for 24 h. Growth experiments were carried out in sterile 50 mL tube containing 10 mL of newly inoculated TSB growth medium and different concentrations (5 and 10%) of LABSA and SLES. The tube was shaken to resububilize the surfactant added. Incubation was performed at 37 °C on a rotary shaker (150 rpm), and optical value of samples were taken at regular intervals by UV–Vis spectrophotometer (Brandt et al. 2001; Turchi et al. 2012).

Growth measurement

Optical density (OD) measurement by using spectrophotometer is a common technique that is used for the measurement

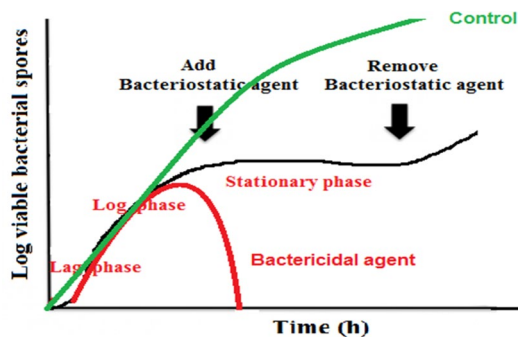


Fig. 1 An illustration for the effects of the bacteriostatic and bactericidal agents

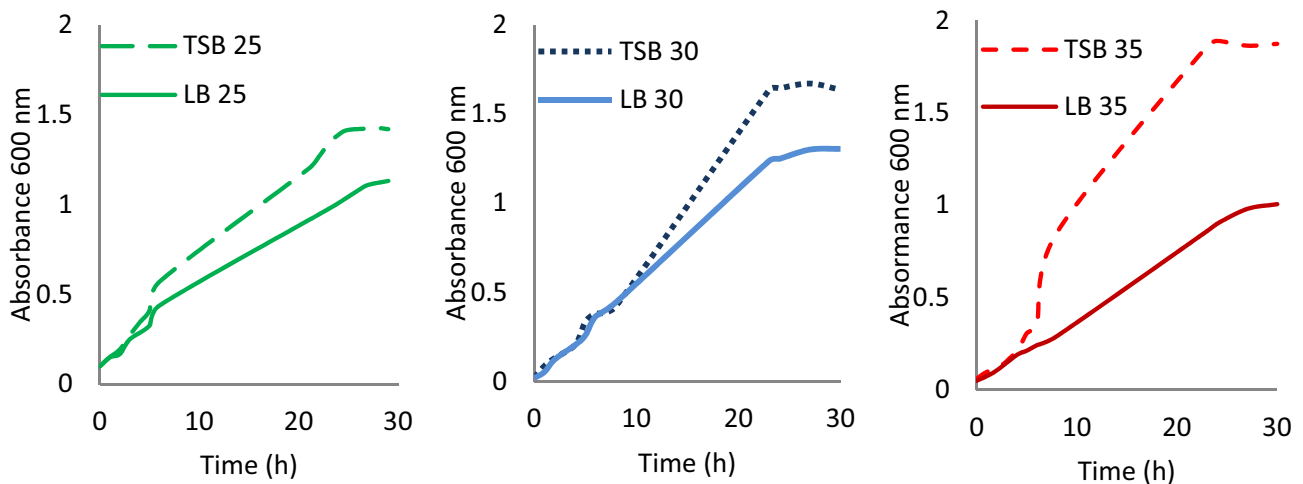


Fig. 2 The effect of LB and TSB liquid medium on growth of *B. subtilis* ATCC 6633

of an exponential bacterial growth curve in batch culture. The changes in optical density were recorded at 1-h intervals at 600 nm by UV–Vis spectrophotometer. The PG Instrument T-60 UV Visible Spectrophotometer device was used for an optical density analysis. The optical density of the bacteria was measured at specific 1-h intervals for 30 h at 600 nm (Arutchelvan et al. 2006).

Results and discussion

Examination of flask cultivation parameters

In the study, the effect of LB and TSB liquid medium and incubation temperature (25 ± 2 , 30 ± 2 and 35 ± 2 °C) on growth of *B. subtilis* ATCC 6633 was examined (Fig. 2). Flask was 1% (v/v) inoculated and incubated on a rotary shaker 150 rpm for 30 h. The optical density of the *B. subtilis* was measured at specific intervals for 30 h at 600 nm (Arutchelvan et al. 2006).

According to Fig. 2, when *B. subtilis* was produced in 35 ± 2 °C TSB liquid medium, it has been observed to show higher growth compared to other temperatures. It was also seen that the lowest growth was achieved at 25 ± 2 °C TSB medium. However, when *B. subtilis* was produced at 35 ± 2 °C in LB liquid medium, it showed the lowest growth compared to other temperatures.

In addition, when *B. subtilis* ATCC 6633 was produced onto TSB liquid medium at each different temperature, it has been observed that it shows higher growth than LB liquid medium. As a result, when the growth curve of *B. subtilis* was examined in Fig. 2, it was observed that *B. subtilis* showed the highest growth when incubated at 35 °C in TSB growth medium, but it showed the lowest growth

when incubated at 35 °C in LB growth medium. In addition, higher growth was achieved when *B. subtilis* growth at different temperatures such as 25 ± 2 , 30 ± 2 and 35 ± 2 °C in TSB medium. However, when incubated at the same temperatures in the LB growth medium, it was observed that it showed a lower growth compared to TSB. In other words, TSB growth medium was observed to be more suitable for the production of *B. subtilis* ATCC 6633.

Determining the growth rates of *B. subtilis* to buffered media

The optimum pH range is different for each type of microorganism used in the production of industrial biotechnological products. Therefore, the pH range and strategy of study should be determined specifically for the microorganism and product (Çalık and Özdamar 2002). To examine cell growth at acidic and alkaline pH, *B. subtilis* was incubated at various pH values from pH 3 to 8 in buffered Luria Bertani (LB) liquid nutrient medium. *B. subtilis* ATCC 6633 was incubated at 25 and 30 °C for 30 h in buffered LB nutrient medium at pH 3, 5, 6, 7, 8, and growth curves were obtained by Optical density (OD) measurement of bacterial cultures (Vehapi and Özçimen 2020).

Bacillus subtilis cells were incubated at 25 and 30 °C for 30 h at various pH values from pH 3 to 8 in buffered LB nutrient medium and growth curves were obtained. *B. subtilis* cells at pH 3, 5, 6 and 8 at 25 and 30 °C showed a long lag time (Fig. 3). In addition, it was observed that when the cells were grown at pH 3, 5, 6 and 8, the adaptation phase lasted longer and *B. subtilis* cells in buffered medium of pH 3 was dead after the adaptation phase. In addition, when the cells were grown at pH 3, 5, 6 and 8, the adaptation phase was observed for the first 4–6 h. On the other hand, when the cells was grown at pH 7, which is the pH value of the medium, it directly adapts to the environment and has passed to the logarithmic phase. After that *B. subtilis* cells in buffered medium of pH 5, 6 and 8 were grown rapidly similar to control sample pH 7. The highest growth was observed

at pH 7 and 8, and the lowest at pH 3 in LB nutrient media at 25 and 30 °C.

A similar lag and dead phases in growth of *B. subtilis* was observed when cultures were incubated at 35 °C in buffered medium from pH 3 to pH 8 in the study of Vehapi and Özçimen (2020). However, in this study, when the temperature was decreased from 35 to 30 °C and 25 °C, higher growth was observed in all pH values except pH 3 (Fig. 3). Faille et al. (2002), in their study, compared pH 5 and 7 and they observed better growth at pH 7 compared to pH 5. Furthermore, when *B. subtilis* cells were grown in pH 5, it showed lower growth compared to pH 8. For this reason, it should be noted that anionic surfactants that used in the probiotic cleaner formulation do not prevent the development of *B. subtilis* cells and do not lower the pH of the cleaner medium (Vehapi and Özçimen 2020).

Spread plate count of *B. subtilis*

B. subtilis spores are promising probiotics and biocontrol agents, which can be used in plants, animals and humans (Chen et al. 2010). After the sporulation of *B. subtilis*, the number of spores was evaluated using spread plate method with serial dilution at 10^{-5} , 10^{-6} and 10^{-7} with PBS (Table 1). Under optimal conditions, typical cell count for *B. subtilis* will be in the range of 1.00×10^8 – 1.52×10^{10} CFU mL⁻¹ as obtained using serial dilution and plate count methods (Posada-Urbe et al. 2015). High spore yields values of *B. subtilis* have been demonstrated in the literature, between 1.00×10^9 spores mL⁻¹– 7.40×10^9 spores mL⁻¹ (Luna et al. 2002; Monteiro et al. 2005).

The number of colonies after *B. subtilis* production at the flask cultivation for 30 h were counted and expressed in CFU mL⁻¹. The number of spores of *B. subtilis* was obtained as high as 4.30×10^8 CFU mL⁻¹. Vehapi and Özçimen (2020) suggested that they achieved 2.7×10^6 CFU mL⁻¹ of spore under small-scale flask cultivation at 35 °C for 30 h onto LB liquid medium. On the other hand, when *B. subtilis* was incubated at 25 and 30 °C in this study, we

Fig. 3 Growth and pH curves of *B. subtilis* in the buffered LB medium and control

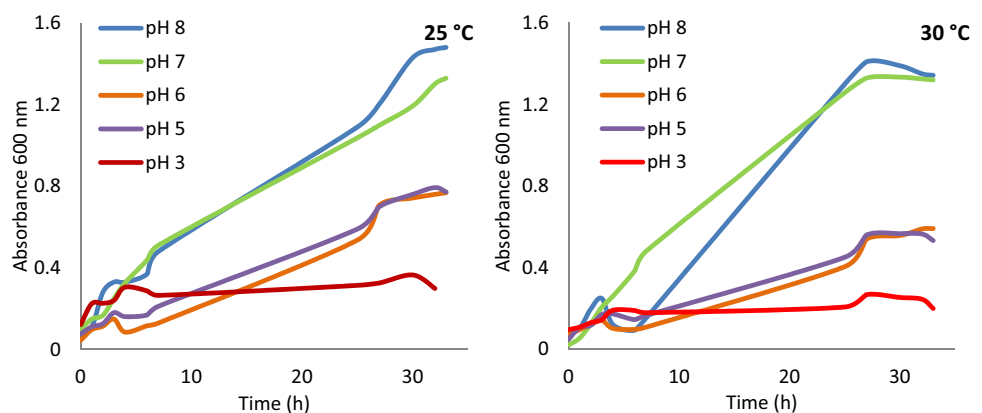


Table 1 Plate count of *B. subtilis* at 10^{-5} , 10^{-6} and 10^{-7} dilution with PBS

Dilutions	10^{-4}	10^{-5}	10^{-6}	10^{-7}	CFU mL ⁻¹	Log CFU mL ⁻¹
Count of flask	TNTC	365	276	98	4.30×10^8	8.63

TNTC too numerous to count

observed that the spore yields of *B. subtilis* were enhanced to 4.30×10^8 CFU mL⁻¹, because higher growth rate of *B. subtilis* was achieved at lower temperature onto LB medium (Table 1). Additional data are given in Online Resource Fig. A. Posada-Urbe et al. (2015) suggested that they achieved 1.37×10^9 CFU mL⁻¹ of spore cell density and 93.5% of sporulation efficiency in shake flask production. Chen et al. (2010) observed the spore yields of *B. subtilis* WHK-Z12 were enhanced to $1.52 \pm 0.06 \times 10^{10}$ and $1.56 \pm 0.07 \times 10^{10}$ spores mL⁻¹ under small-scale flask cultivation and 30 L fermenter fermentation conditions, respectively.

Antagonistic activity of *B. subtilis*

After obtained 10^8 CFU mL⁻¹ viable cells from *B. subtilis*, 0.01 mg weighed and dissolved with 1 mL of phosphate buffered saline (PBS) to adjust at 10^6 CFU mL⁻¹, and antagonistic effect of *B. subtilis* cells were examined with agar disc diffusion method against *B. cereus*, *S. aureus*, *K. pneumoniae* and *S. typhimurium* indicator species (Table 2).

As a result, *B. subtilis* inhibited indicator *B. cereus*, *S. aureus*, *K. pneumoniae* and *S. typhimurium* species and showed germination in the environment. Germination of *B. subtilis* was measured for 3 days (Table 2). Lertcanawanichakul and Sawangnop (2008) suggested that most *Bacillus* species exhibited inhibitory activity after 72 h of incubation and remained up to the 5th day and they observed that, *S. aureus* was more sensitive than *E. coli*. In addition, the most sensitive bacterium in this study was observed as *S. aureus* and *K. pneumoniae* with 22.50 and 24.00 mm germination zone of *B. subtilis*, respectively. It has been observed that *B. subtilis* continues to germinate as long as it remains in the nutrient medium (Table 2). The results obtained from

agar disc diffusion showed that, *S. aureus*, *K. pneumoniae* and *S. typhimurium* were more resistant than *B. cereus* to probiotic *B. subtilis*. Lee et al. (2010) suggested that they isolated BSAP-254 from *B. subtilis* SC-8 and they showed antagonistic activity against gram-positive bacteria *B. cereus* with inhibition zone smaller than 10 mm.

Antimicrobial activity of surfactant against *B. subtilis*

Antimicrobial effects of various surfactants and other additives were evaluated against *B. subtilis* to be used in probiotic-based cleaner formulation. The antimicrobial activity of additives such as, PEG 40 hydrogenated castor oil, benzo-phenone, citric acid, isopropyl alcohol (IPA), monopropylene glycol, triethanol amine (TEA), and nonionic, anionic and amphoteric surfactants were investigated for probiotic cleaner formulation.

Table 3 shows that growth of *B. subtilis* was inhibited with anionic, nonionic and amphoteric surfactants but was not inhibited by other additives used for cleaner formulation. Therefore, an “inhibition zone” is measured to determine the susceptibility of microorganism to surfactants. It is then classified as following microorganisms: Resistant (R), Intermediate (I) or Sensitive (S) (Table 3) (Bauer et al. 1966).

Surfactants used in the cleaning composition containing probiotics should not have any adverse effect on *B. subtilis* (Brooke 2014). The purpose of this study was to determined surfactants for use in formulation of cleaning composition with a lower antimicrobial effect against *B. subtilis*.

The probiotic cleaning composition may also contain additives. These additives are viscosity modifiers, fragrances, foaming agents, water softening agents, preservatives, stabilizing agents, anionic, cationic, nonionic,

Table 2 The antagonistic activity of *B. subtilis* spores against *B. cereus*, *S. aureus*, *K. pneumoniae* and *S. typhimurium*

Incubation period (days)	Inhibition zone (mm)			
	<i>B. cereus</i>	<i>S. typhimurium</i>	<i>S. aureus</i>	<i>K. pneumoniae</i>
1	$10.00 \pm 0.00^{A,a}$	$11.50 \pm 0.70^{B,a}$	$11.00 \pm 0.00^{B,a}$	$13.75 \pm 0.35^{C,a}$
2	$10.00 \pm 0.00^{A,a}$	$15.00 \pm 0.00^{B,b}$	$16.50 \pm 0.70^{C,b}$	$19.00 \pm 1.41^{D,b}$
3	$10.00 \pm 0.00^{A,a}$	$16.00 \pm 0.00^{B,c}$	$22.50 \pm 0.70^{C,c}$	$24.00 \pm 0.00^{D,c}$

Data are presented as means \pm standard deviations ($n=3$) ($p < 0.05$)

A–D: Within each row, different superscript uppercase letters show differences between the type of pathogenic bacteria

a–c: Within each column, different superscript lowercase letters show differences between the each storage period

Table 3 Antimicrobial effect of various surfactants and additives against *B. subtilis*

Surfactants and additives	Inhibition zone (mm)	Susceptibility
Polyethylene glycol (PEG) 40	NZ	R
APG-nonionic	22.50 ± 3.53	S
Benzophenone	NZ	R
Citric acid	NZ	R
Fragrance	NZ	R
Isopropyl alcohol (IPA)	NZ	R
Nonionic active matter 1	26.00 ± 4.24	S
Mono propylene glycol	05.00 ± 7.07	R
Anionic active matter 1	29.50 ± 0.70	S
Triethanol amine (TEA)	20.50 ± 7.77	S
Anionic active matter 2	19.05 ± 0.35	I
Nonionic active matter 2	19.26 ± 1.06	I
Amphoteric active matter	21.00 ± 0.00	S
Cocamide Dea	19.15 ± 2.47	I

NZ no inhibition zone

Data were presented as means with ± standard deviations ($n=3$)

amphoteric and zwitterionic surfactants, additional organic solvents for physical stability, sequestering agents (chelating agents), amino carboxylate chelating agents (EDTA and metal salts), carboxylate chelating agents, aromatics and mixtures thereof and coloring agents (Brooke 2014). Citric acid has been used in cleaning products due to its cleaning properties (Kapiloff and Hatch 1985). These optional components should be chosen so that they have little or no negative impact on *Bacillus* species (Brooke 2014). As a result, it is observed that nonionic surfactants are more compatible with *B. subtilis* (Table 3). Additional data are given in Online Resource Fig. B.

The bactericidal action of surfactants increases with the number of carbon atoms in the fatty acid, usually highest in those soaps containing 12 or 14 carbon atoms. Cowles (1938) also concluded that for these straight chain alkyl

sulphates, there is a relationship between chain length and inhibition against *Staphylococcus aureus*, and the optimum chain length is between 12 and 16 carbon atoms (Cowles 1938). Birkeland and Steinhaus (1939) was concluded that sodium lauryl sulfate and “Dreft” inhibit the growth of Gram-positive bacteria and molds, but almost all Gram-negative bacteria grow freely. In et al. (2013) described that the growth of *S. sonnei* was not inhibited by 1–3% citric acid, but the growth of other species *S. flexneri*, *S. boydii* and *S. dysenteriae* was weakly inhibited by >2% citric acid. Cowles (1938) mentioned that *E. coli* is able to grow in a concentration of 5% sodium lauryl sulfate.

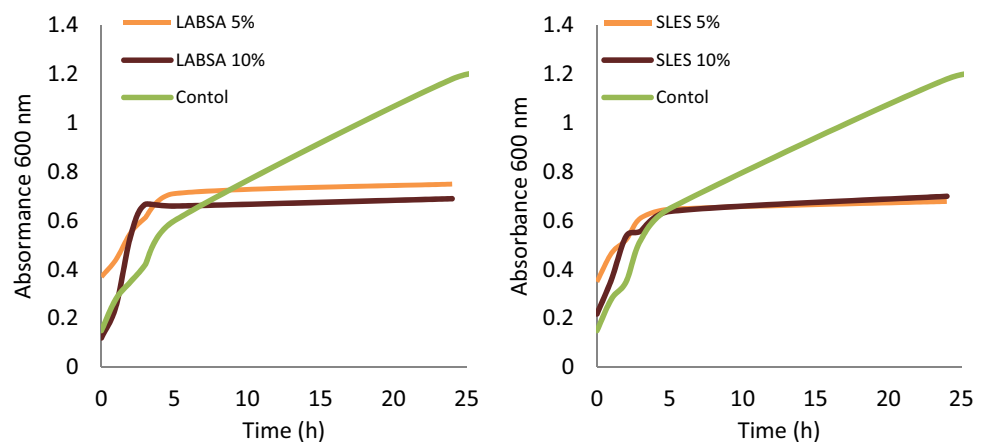
Effects of LABSA and SLES on growth in liquid cultures

The inhibiting action of varying quantities of the different alkyl sulfates was tried on *B. subtilis* only. TSB broth was inoculated with single colony *B. subtilis* culture and after 2 h of incubation 5 and 10% dilutions of the anionic surfactants were added in the same medium (Fig. 4).

So, anionic surfactants (LABSA and SLES) at varying concentrations were added at 2 h into TSB medium that containing *B. subtilis*. For this, *B. subtilis* inoculated into tryptic soya broth (TSB), and after 2 h of inoculation (OD = 0.5) the LABSA and SLES surfactants were added onto TSB liquid medium. Results were recorded at 1-h intervals by spectrophotometer at 600 nm for 24-h incubation at 37 °C.

The inhibiting action of varying anionic surfactants was tried on *B. subtilis*. When cultures were treated with anionic surfactants (LABSA and SLES), exponential growth phase was followed by a stationary phase and cell growth had passed to the stationary phase (Fig. 4). Cowles (1938) has been found that Gram-positive bacteria are generally inhibited by the concentrations of alkyl sulfate used, but Gram-negative bacteria (except two species) are not. In fact, it has been observed that *Escherichia coli* can withstand much higher concentrations of two surfactants compared

Fig. 4 Growth curves of the *B. subtilis* in liquid medium containing different SLES and LABSA concentration



to other Gram-negative bacteria (Cowles 1938). Similarly, Birkeland and Steinhaus (1939) investigated the growth inhibitory properties of a wide range of Gram-positive and Gram-negative bacteria of sodium lauryl sulfate (sodium dodecyl sulfate) and the commercial product “Dreft” (containing large amounts of alkyl sulfate). Brandt et al. (2001) described that growth of all four AOB strains was progressively inhibited as the LAS concentration was increased.

In the absence of anionic surfactants, (LABSA and SLES) *B. subtilis* (control) showed exponential growth from inoculation until the stationary phase was reached after approximately 24 h. On the other hand, anionic surfactant (LABSA and SLES)-treated samples were passed to the stationary phase when the anionic surfactants added to culture medium at 2 h. In this study, bacteriostatic action of sodium lauryl ether sulfate (SLES) and linear alkyl benzene sulfonic acid (LABSA) which have common use as hard surface detergents during the past few years (Cowles 1938; Caselli 2017; D’Accolti et al. 2018; De Cesare et al. 2019) were presented. The *B. subtilis* also showed immediate, exponential growth until the LABSA and SLES were added to the culture medium. Nevertheless, *B. subtilis* exhibited growth rates similar to those recorded at all surfactant levels. Also in these strains, the LABSA and SLES addition resulted in complete inhibition of growth or at least in reduced initial growth rates. Cowles (1938) mentioned that *Bacillus* species such as *B. anthracis*, *B. megatherium*, *B. mesentericus*, *B. subtilis* and *S. aureus* was not able to grow in a concentration of 0.1 and 0.03% sodium lauryl sulfate.

Conclusion

As a result, it was observed that the *B. subtilis*, which can be used in the detergent formulation, shows higher growth and viability at the neutral pH, and it passes into the death phase at pH 3. For this reason, surfactants and other additives used in the probiotic cleaner formulation do not prevent the development of *B. subtilis* and do not lower the pH of the medium (Vehapi and Özçimen 2020).

The antagonistic effects of *B. subtilis* against Gram-positive *S. aureus* and *B. cereus* and Gram-negative *K. pneumoniae* and *S. typhimurium* were evaluated by agar disk diffusion method. The antagonistic activity methods showed that the Gram-positive *S. aureus* and *K. pneumoniae* were the most sensitive bacteria while *B. cereus* was the most resistant bacteria. The antimicrobial compounds, produced by *Bacillus* strains, have a great potential for controlling the growth of Gram-positive and Gram-negative bacteria (Lertcanawanichakul and Sawangnop 2008; Todorova and Kozhuharova 2010; Moore et al. 2013).

The probiotic cleaning composition also includes one or more nonionic, anionic, cationic, amphoteric and zwitterionic surfactants or mixtures thereof. Surfactants used in the cleaning composition, containing probiotics, should not have any negative effects on *Bacillus* species (Vehapi and Özçimen 2020). The probiotic cleaning composition can comprise 0.1–35% by weight of anionic and/or non-ionic surfactants (Brooke 2014). Anionic surfactants are an amine salt of alkylbenzene sulfonic acid and alkyl ether sulfate (Cowles 1938).

The probiotic cleaning composition may also contain additives. Such additives are viscosity modifiers; fragrances; foaming agents; water softening agents; preservatives; stabilizing agents; additional partners include anionic, cationic, nonionic, amphoteric and zwitterionic surfactants; additional organic solvents for physical stability; sequestration agents (chelating agents); amino carboxylate chelating agents (EDTA and their metal salts); carboxylate chelating agents; aromatic substances and mixtures thereof and coloring agents. These optional ingredients should be chosen so that they have little or no negative impact against *Bacillus* species (Brooke 2014).

Therefore, it should be concluded that surfactants used in the microbial based cleaner formulation does not prevent the development of *B. subtilis* and does not lower the pH of the medium (Vehapi and Özçimen 2020). Consequently, the LABSA and SLES may be preferred for the probiotic cleaner formulation due to the fact that they act as bacteriostatic on *Bacillus* cells and do not cause cell death.

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Declarations

Conflict of interest The authors declare that no conflicts of interest exist.

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